Tick-Borne Zoonotic Bacteria in Ticks Collected from Central Spain

Álvaro Toledo, A. Sonia Olmeda, Raquel Escudero, Isabel Jado, Félix Valcárcel, Miguel A. Casado-Nistal, Manuela Rodríguez-Vargas, Horacio Gil, and Pedro Anda*

Laboratorio de Espiroquetas y Patógenos Especiales. Servicio de Bacteriología, Centro Nacional de Microbiología, Instituto de Salud Carlos III. Majadahonda, Madrid, Spain; Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain; Universidad Alfonso X ‘El Sabio,’ Villanueva de la Cañada, Madrid, Spain

Abstract. The prevalence of tick-borne and related bacteria infecting adult ticks in central Spain was assessed by molecular methods. Six areas were sampled monthly during a 2-year longitudinal study. A total of 1,038 questing and 442 feeding ticks, belonging to eight different species, were tested. The most abundant species were Hyalomma lusitanicum (54% of captures), followed by Dermacentor marginatus (23%) and Rhipecephalus sanguineus (10%). Four human pathogens, including seven Rickettsia species, Anaplasma phagocytophilum, Borrelia burgdorferi, and Francisella tularensis, were detected at percentages of 19.0, 2.2, 1.7, and 0.5, respectively, whereas Bartonella spp. was never detected. In terms of infection and tick abundance, H. lusitanicum seems to be the most significant tick species in the area, carrying three of the five agents tested, and the anthropophilic tick, D. marginatum, infected with Rickettsia spp. and F. tularensis, is the most relevant in terms of public health. The significance of these data is discussed.

INTRODUCTION

A wide range of tick-borne agents, including protozoa, viruses, and bacteria, causes diseases in humans and animals worldwide. Ticks are considered, after mosquitoes, the most important vectors for infectious diseases worldwide, and the interest in tick-borne diseases has increased in recent years. Also, ticks play an important role in the maintenance of these pathogens, including seven Rickettsia species, Anaplasma phagocytophilum, Borrelia burgdorferi, and Francisella tularensis, are recognized as potential agents of bioterrorism.2,3 Borrelia spp. and Anaplasma phagocytophilum complete the list of recognized tick-borne bacteria of medical interest worldwide. Additionally, although Bartonella spp. are not considered specifically tick-borne pathogens, they have been largely detected in ticks, and successful experiments of transmission of Bartonella henselae in Isodes ricinus have been recently described,4,5 the determination of their prevalence in ticks being of interest.9

Previous studies performed in different areas of Spain have shown the presence of several tick-borne pathogens among ixodid populations. In the western and northern fringes of the country, A. phagocytophilum, Borrelia burgdorferi s.l., and spotted fever group (SFG) Rickettsia species were found in ticks.10–18 However, there are few data about the prevalence of such pathogens in central Spain.19 Climatic differences among different areas of Spain are notorious and are responsible for the diversity of tick species observed between and within them (A. L. García-Pérez and others, unpublished data) and, consequently, of the circulation of tick-borne pathogens. To provide a preliminary risk assessment for tick-borne bacterial diseases for humans and animals based on their potential exposure to ticks in central Spain, questing and feeding adult ticks were analyzed by molecular methods to determine the infection rate of A. phagocytophilum, Bartonella spp., Borrelia spp., F. tularensis, and Rickettsia spp.

MATERIALS AND METHODS

Tick collection and identification. Ticks were collected monthly from vegetation in six areas in central Spain (five in Madrid and one in Toledo) by the blanket-dragging method described by Sonenshine.20 Briefly, a 2 × 1.6-m white blanket was dragged for 30 minutes, and ticks attached to the blanket were collected every 5 minutes. Also, ticks feeding on domestic or wild animals were manually collected from 17 host species in adjacent areas (Table 1). All specimens were identified to the species level by using appropriate taxonomic keys.21,22

DNA extraction and polymerase chain reaction assays. Tick were individually crushed with a plastic homogenizer (ICN Biomedicals, Irvine, CA), and the resultant material was processed using the QIAamp DNA blood mini kit (QIAGEN, Hilden, Germany) for DNA extraction, with a previous overnight treatment with a proteinase K solution (20 mg/mL), and a final step of 15 minutes at 100°C for inactivation of the protease. Water was included as a negative control every 20 samples to test possible contaminations. The concentration of DNA was measured by using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), and between 200 and 400 ng of DNA from each sample were subjected to polymerase chain reaction (PCR).

Two sets of duplex PCR were set up to amplify any species of Borrelia and Rickettsia on the one hand and Bartonella and Francisella on the other hand (Table 2 and references therein). PCRs were performed in a volume of 50 μL, with 10 mmol/L Tris-HCl, 50 mmol/L KCl, 2 mmol/L Cl-Mg, 200 μmol/L of each deoxynucleoside triphosphate (Promega, Madison, WI), 2.5 U of Taq Gold DNA polymerase (Applied Biosystems, Branchburg, NJ), and 0.8 μg/μL of DNase-free bovine serum albumin (General Electric Healthcare Spain, Barcelona, Spain), with primers used at a final concentration of 0.5 μmol/L. PCR cycling included an initial denaturing step of 9 minutes at 94°C, followed by 40 cycles of 15 seconds at 94°C, 1 minute at 60°C, and 4 minutes at 65°C, with a final elongation of 7 minutes at 65°C in an MJ Research PCT-200 (Ecogen, Barcelona, Spain), as previously described.23 A third single PCR was used for the detection of A. phagocytophilum msp2 as described.24

The prevention of cross-contamination and false-positive results was managed by using plugged tips, setting PCRs in a room separate from that used for DNA extraction, and reserv-
ing specific separated areas for the production of reagents and analyses of the amplicons. A negative PCR control (water) was included in each run as well.

Identification of positives by RLB hybridization and sequencing. To increase the detection limit and the specificity of the PCR assays, PCR amplicons were hybridized to probes specific for *A. phagocytophilum*, *Bartonella* spp., *Francisella* spp., *Rickettsia* spp., and *Rickettsia* spp. (Table 2 and references therein) by reverse line blot (RLB) performed as previously described with minor modifications as follows: 3.2 pmol/μL of each probe were attached to the membrane; the hybridization was performed at 52°C for 1 hour; and the washing steps were done at 48°C. The sensitivity of the methods was calculated by amplifying 10^3, 10^2, 10, and 1 genome equivalents (GEs) (in the case of *B. henselae*, *B. burgdorferi*, and *F. tularensis*) or plasmid copies (PCs) with inserts of synthetic DNA (in the case of *A. phagocytophilum* and *R. bellii*) in spiked distilled water as well as in pathogen-free *I. ricinus* DNA. Synthetic DNA used as positive controls (for *R. bellii* and *A. phagocytophilum*) was calculated as previously described by using overlapping primers up to 72 bp in length in consecutive PCRs.

Samples positive to *Rickettsia* spp. were further analyzed using species-specific probes for a wide range of SFG *Rickettsia* spp., including *R. aeschlimanii*, *R. australis*, *R. bellii*, *R. conorii*, *R. felis*, *R. helvetica*, *R. rickettsii*, and *R. slovaca*, as previously described. Additional probes for this method were designed in this study for *R. massiliae*, *R. sibirica*, and *R. raoultii*, following the same methodology. In a representative number of positives, specificity of the hybridization was tested by sequencing *ompA* or *gltA* as described.

For *Borrelia*, all the positives were subjected to PCR and subsequent RLB targeting the 5S-23S rRNA intergenic spacer for genospecies determination, as described. *F. tularensis* subspecies determination was accomplished by sequencing a fragment of TUL4 (*lpnA*), as described.

All oligonucleotides used are specified in Table 2.

Statistical analysis. Prevalence for each bacterial species was analyzed according to independent variables such as sampling site, biological origin of the specimens (questing or feeding), and tick species by χ² or Fisher exact tests using the SPSS statistical package (version 15.0; SPSS Ibérica, Madrid, Spain). Significance was set at *P* < 0.05.
**GenBank accession numbers.** Sequences generated in this study were deposited in GenBank under accession numbers FJ151534–FJ151540 for *F. tularensis*; FJ157299–FJ157303 for *R. aeschlimannii*; FJ157304–FJ157308 for *R. massiliae*; FJ157309–FJ157314 for *R. slovaca* strain mongolotimonae; FJ157315–FJ157319 for *R. sibirica*; FJ157320–FJ157324 for *R. bellii*; FJ157299–FJ157303 for *R. sanguineus* sp. strain RpA4; FJ157325 for *R. felis*; and FJ157326 for *R. helvetica*.

**RESULTS**

**Tick collection.** A total of 1,480 adult tick specimens, 1,038 questing (QT) and 442 ticks feeding on animals (TA), were collected, and they belonged to eight species of five different genera (Table 3). The most frequently found species was *Hyalomma lusitanicum*, with 795 specimens collected (701 QT and 94 TA), followed by *Dermacentor marginatus* (348 specimens: 265 QT and 83 TA). Other species found in both vegetation and animals were *Rhipicephalus pusillus* and *R. bursa* (108 and 56 specimens, respectively), whereas *R. sanguineus* and *H. marginatum* (146 and 13 specimens, respectively) were only collected from animals. Ticks from animals were removed from livestock represented 0.9%, 0.5%, and 0.4% of the ticks collected, respectively. Ticks from animals were removed from livestock (185 specimens), pets (116), wild mammals (110), lagomorphs (28), and birds (3) (data not shown).

**Prevalence of tick-borne bacteria in ticks.** The detection limit for the combined PCRs and subsequent hybridizations was between 10 (for *A. phagocytophilum*, *B. burgdorferi*, and *R. bellii*) and 100 (for *B. henselae* and *F. tularensis*) GEIs or PCs, without any loss in sensitivity when amplifying spiked positive controls with 300 ng of pathogen-free *I. ricinus* DNA (data not shown). Also, all the nucleotide probes used for the hybridization assays gave positive results with their corresponding positive controls and did not show cross-reactions.

Overall, the prevalence of infection found in ticks was 22.9% (20.5 for QT and 28.5 for TA). It was different among tick species and bacteria analyzed (Table 3). A percentage of 5.1 of *H. lusitanicum* (41/795) was found infected. This percentage for the rest of species was 66.7 for *D. marginatus* (232/348), 26.0 for *R. sanguineus* (38/146), 6.5 for *R. pusillus* (7/108), 17.8 for *R. bursa* (10/56), 61.5 for *R. sibirica* strain mongolotimonae; and 37.5 for *I. ricinus* (3/8). None of the five specimens of *H. hispanica* were found to be infected (Table 3).

*Rickettsia* spp. DNA was detected in 19.0% of ticks (281/1,480) from five of the six areas sampled (Figure 1), and all of these hybridized with the specific probe for SFG. This percentage was of 15.9 and 26.2 for QT and TA, respectively, a difference that was statistically significant (*P* < 0.05). Of the 281 positives, 8 also hybridized with one of the specific probes used in the RLB, including 4 *R. pusillus* and 2 *R. bursa* with P-SIB (for *R. sibirica*), 1 *R. sanguineus* with P-FEL (for *R. felis*), and 1 *I. ricinus* with P-HELV (for *R. helvetica*). In all these samples, a fragment of *rOmpA* (*gltA* in the case of *R. helvetica*) was sequenced, and the results confirmed an identity of 100% with the corresponding species (data not shown). In the

---

**Table 3. Results of RLB analysis in different tick species**

<table>
<thead>
<tr>
<th>Tick species (percentage of positives)</th>
<th>QT</th>
<th>TA</th>
<th>QT</th>
<th>TA</th>
<th>QT</th>
<th>TA</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>H. lusitanicum</strong></td>
<td>701</td>
<td>94</td>
<td>265</td>
<td>83</td>
<td>146</td>
<td>47</td>
<td>37.5</td>
</tr>
<tr>
<td><strong>D. marginatus</strong></td>
<td>14(1.8)</td>
<td>0</td>
<td>4(2.4)</td>
<td>0</td>
<td>2(0.7)</td>
<td>0</td>
<td>0.4%</td>
</tr>
<tr>
<td><strong>R. sanguineus</strong></td>
<td>2(0.7)</td>
<td>0</td>
<td>4(2.4)</td>
<td>0</td>
<td>1(0.4)</td>
<td>0</td>
<td>0.7%</td>
</tr>
<tr>
<td><strong>R. bursa</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.5%</td>
</tr>
<tr>
<td><strong>R. sibirica</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.6%</td>
</tr>
<tr>
<td><strong>I. ricinus</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.6%</td>
</tr>
<tr>
<td><strong>R. marginatus (100%)</strong></td>
<td>158(93.6)</td>
<td>0</td>
<td>64(71)</td>
<td>0</td>
<td>31(32.5)</td>
<td>0</td>
<td>37.5</td>
</tr>
<tr>
<td><strong>R. sanguineus (70%)</strong></td>
<td>37(53.3)</td>
<td>4(4.2)</td>
<td>166(62.6)</td>
<td>66(79)</td>
<td>38(67)</td>
<td>3(18)</td>
<td>22.9</td>
</tr>
<tr>
<td><strong>Total infections for each genus</strong></td>
<td>19</td>
<td>0</td>
<td>25</td>
<td>5</td>
<td>25</td>
<td>4</td>
<td>22.9</td>
</tr>
<tr>
<td><strong>Number of infection positive ticks</strong></td>
<td>232</td>
<td>0</td>
<td>252</td>
<td>0</td>
<td>19</td>
<td>2</td>
<td>9.5%</td>
</tr>
<tr>
<td><strong>A. phagocytophilum</strong></td>
<td>25</td>
<td>0</td>
<td>25</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>1.6%</td>
</tr>
<tr>
<td><strong>B. burgdorferi</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td><strong>F. tularensis</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td><strong>Rickettsia sp.</strong></td>
<td>2(0.7)</td>
<td>0</td>
<td>4(2.4)</td>
<td>0</td>
<td>1(0.4)</td>
<td>0</td>
<td>0.7%</td>
</tr>
<tr>
<td><strong>Total single and mixed infections</strong></td>
<td>37</td>
<td>4</td>
<td>166</td>
<td>66</td>
<td>38</td>
<td>3</td>
<td>22.9</td>
</tr>
</tbody>
</table>

Numbers in parentheses represent the percentages of positive ticks from the total number of ticks analyzed. QT = questing ticks; TA = ticks collected from animals.
case of the six samples positive to P-SIB, the results confirmed that *R. sibirica* (strain mongolotimonae) was the organism present in the samples. The remaining 273 positives reacted with more than one probe. Given the high homology in the 23S-SS sequence among *R. aeschlimanii*, *R. massiliae*, *R. slovaca*, and *R. raoultii*, a pattern of reactivity against five probes (P-AESCH, P-MAS, P-RPA4, P-SIB, and P-SLO) was used for their classification. When using native positive controls (*R. aeschlimanii*, *R. massiliae*, *R. slovaca*, and *R. sibirica* strain mongolotimonae), *R. aeschlimanii* reacted with P-AESCH and P-RPA4, *R. massiliae* reacted with P-MAS, P-RPA4, and P-SIB, and *R. slovaca* reacted with P-SLO, P-RPA4, and P-SIB. The reproducibility of this approach was confirmed in different assays. These patterns were observed in different samples, along with an additional one that showed reactivity with P-RPA4 and P-SIB and was associated with specimens carrying *R. raoultii*. To confirm the *Rickettsia* species present in each sample, the same fragment of *rOmpA* as before was sequenced in 20 samples, representing five positives within each of the four patterns, and the results confirmed the specificity of this approach (data not shown). An example is shown in Figure 2 and a summary of the results in Table 4. Taking this into account, from the 273 positives that reacted with more than one probe, 8 corresponded to *R. aeschlimanii*, 40 to *R. massiliae*, 188 to *R. raoultii*, and 37 to *R. slovaca* (Table 4).

During the study, *R. aeschlimanii–*infected feeding ticks were all removed from livestock (*H. marginatum* from horses and cattle), whereas *R. sibirica* (strain mongolotimonae) was found in *R. bursa* removed from cattle and sheep and in *R. psilus* from rabbits. The only tick positive to *R. felis* was an *R. sanguineus* specimen collected from a dog. The rest of the *Rickettsia*-infected ticks collected from animals were mostly *R. raoultii–*infected *D. marginatus*, removed from wild boars, horses, deer, cattle, and sheep, and *R. massiliae–*infected *R. sanguineus* and *R. psilus*, removed from foxes, hedgehogs, beech martens, and pets (*R. massiliae*–infected *R. sanguineus* from dogs; data not shown).

*Anaplasma phagocytophilum* was the second most frequently found agent, being detected in 32 (2.2%) ticks (25 QT and 7 TA) from five of the six areas studied (Figure 1). Statistically significant differences were not observed between QT and TA (*P* > 0.05). The largest number of positives corresponded to *H. lusitanicum* (23/795), followed by *D. marginatus* (4/349), *R. bursa* (4/56), and *R. psilus* (1/108). No positive

**Figure 1.** Map of the Madrid region showing the sampling areas. Circles represent each bacterial genus studied and found in each area. A, *A. phagocytophilum*; B, *B. burgdorferi*; F, *F. tularensis*; R, *Rickettsia* spp.

**Figure 2.** Pattern of reactivity of different *Rickettsia* species against a panel of probes for the identification of four *Rickettsia* species/strains. **Left**, pattern of reactivity of different *Rickettsia* species against a panel of probes. **Right**, representative examples of the hybridization of tick species carrying different *Rickettsia*. Lanes 1 and 2, *D. marginatus* specimens positive to *R. slovaca*; Lane 3, *D. marginatus* positive to *Rickettsia* sp. strain RPA4; Lane 4, *R. pusillus* positive to *R. sibirica*, mongolotimonae strain; Lanes 5 and 6, *H. marginatum* positive to *R. aeschlimanii*; Lanes 7–9, *R. sanguineus* positive to *R. massiliae*. The names of the reactive probes are shown on the left. GP-RICK, *Rickettsia* catch-all; GP-SFG, spotted fever group *Rickettsia* catch-all; P-MAS, probe for *R. massiliae*; P-Rpa4, probe for the strain RPA4; P-SIB, probe for *R. sibirica*, mongolotimonae strain; P-SLO, probe for *R. slovaca*.

**Table 4**

Identification of *Rickettsia* species in the samples studied

<table>
<thead>
<tr>
<th>Tick species</th>
<th>No. studied</th>
<th><em>R. aeschlimanii</em></th>
<th><em>R. felis</em></th>
<th><em>R. helvetica</em></th>
<th><em>R. massiliae</em></th>
<th>Rpa4 strain</th>
<th><em>R. sibirica</em> mongolotimonae</th>
<th><em>R. slovaca</em></th>
<th>Total positives</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. lusitanicum</em></td>
<td>795</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>D. marginatus</em></td>
<td>348</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>186 (53.4)</td>
<td>0</td>
<td>37 (10.6)</td>
</tr>
<tr>
<td><em>R. sanguineus</em></td>
<td>146</td>
<td>0</td>
<td>1 (0.7)</td>
<td>0</td>
<td>36 (24.6)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>37 (25.3)</td>
</tr>
<tr>
<td><em>R. pusillus</em></td>
<td>108</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2 (1.8)</td>
<td>0</td>
<td>4 (3.7)</td>
<td>0</td>
<td>6 (5.5)</td>
</tr>
<tr>
<td><em>R. bursa</em></td>
<td>56</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2 (3.6)</td>
<td>2 (3.6)</td>
<td>2 (3.6)</td>
<td>0</td>
<td>6 (10.7)</td>
</tr>
<tr>
<td><em>H. marginatum</em></td>
<td>13</td>
<td>8 (61.5)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8 (61.5)</td>
</tr>
<tr>
<td><em>I. ricinus</em></td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>1 (12.5)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (12.5)</td>
</tr>
<tr>
<td><em>H. hispanica</em></td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>1,480</td>
<td>8 (0.5)</td>
<td>1 (0.06)</td>
<td>1 (0.06)</td>
<td>40 (2.7)</td>
<td>188 (12.7)</td>
<td>6 (0.4)</td>
<td>37 (2.5)</td>
<td>281 (19.0)</td>
</tr>
</tbody>
</table>

Percentages over total studied are in parentheses.
specimens of *I. ricinus* were found. *Anaplasma*-positive feeding ticks were all collected from livestock (four *H. lusitanicum* from cattle and three *R. bursa* from a cow and two sheep).

*Borrelia* spp. was detected in 25/1,480 specimens (1.7%) from four areas (Figure 1). The positives were only found among questing ticks from two different species: *H. lusitanicum* (23/701 specimens, 3.3%) and *I. ricinus* (2/8, 25%). No ticks collected from animals were found to be positive; this difference was statistically significant (*P* < 0.05). *B. burgdorferi* genospecies were determined in 18 of the 25 positives; 14 *B. burgdorferi* s.s. (12 specimens of *H. lusitanicum* and 2 specimens of *I. ricinus*), 3 *B. garinii* (from *H. lusitanicum*), and a mixed infection with *B. burgdorferi* s.s. and *B. garinii*, also detected in *H. lusitanicum*. For the rest of the positives obtained (seven specimens of *H. lusitanicum*), the hybridization signal was very faint for the generic probe for *B. burgdorferi* s.l., and no hybridization was detected against the specific probes nor were attempts to sequence these samples successful.

Francisella tularensis DNA was detected in seven (0.5%) ticks from three different species: *D. marginatus* (five specimens), *H. lusitanicum* (one specimen), and *R. sanguineus* (one specimen). The positives were collected from two of the six areas tested that are geographically close (Figure 1). *D. marginatus* was the only species found to harbor DNA from *F. tularensis* in both QT and TA, whereas the only positive specimen of *H. lusitanicum* was collected from vegetation. Statistically significant differences were not observed between QT and TA (*P* > 0.05). All the positives were sequenced and corresponded to *F. tularensis* subsp. *holarctica*. The three specimens of Francisella-infected ticks found feeding on animals were removed from a horse (two *D. marginatus*) and a dog (one *R. sanguineus*).

No positives to *Bartonella* were found, and mixed infections with bacteria from different genus accounted for 0.5% of ticks (7/1,480) and included *A. phagocytophilum* and *B. burgdorferi*, found in six specimens of QT *H. lusitanicum* (0.8%), and *F. tularensis* and *R. raoultii*, found in one specimen of QT *D. marginatus*.

**DISCUSSION**

The results presented here represent the first systematic study of tick abundance and tick-borne pathogens performed in central Spain. Regarding tick species abundance, our results differ from those obtained by other authors. The most frequently found tick species was *H. lusitanicum*, which accounted for 54% of captures, whereas in other areas such as the Basque Country, this was *I. ricinus*. The relative abundance of the other seven tick species is evidence of the high diversity of ixodids in the study area.

We confirmed that *Rickettsia* infection is common in ticks from central Spain. In fact, the presence of *Rickettsia* was detected in six of the eight tick species found in the area, with the exception of *H. lusitanicum* and *H. hispanica*. Significant differences were observed between the percentage of *Rickettsia* infection in ticks from vegetation (15.9%) and ticks feeding on animals (26.2%), suggesting that ticks are feeding on competent reservoirs. The lack of infection by *Rickettsia* in *H. lusitanicum*, of which a large number of specimens were tested, could be related to a vectorial incompetence of such tick species for the *Rickettsiae* that are circulating in the area. Most of the positives were detected in *D. marginatus*, of which the majority corresponded to *R. raoultii* (53.4% of the *D. marginatus* tested), *R. slovaca* being the second most frequently found species in that ixodid species (10.6%). The same situation has recently been described in Portugal and in ticks collected from wild boar and Iberian red deer in a different area of central Spain. *R. raoultii* has been suspected to be a second etiologic agent of TIBOLA, after *R. slovaca*. Consequently, whether the pathogenicity of this *Rickettsia* is confirmed or not, the risk of developing TIBOLA in persons bitten by a *D. marginatus* in central Spain seem to be high, according to this data.

We found *R. aeschlimannii* infecting 8 of 13 specimens of *H. marginatum*. The presence of *R. aeschlimannii* in *Hyalomma* spp. has recently been reported in other southern European countries such as Croatia and Greece, apart from Spain, where it was detected also in additional tick species such as *I. ricinus*, *R. sanguineus*, *R. turanicus*, *R. bursa*, and *H. punctata*. In contrast, in this study, this agent was only identified in *H. marginatum* specimens, the only tick species from which *R. aeschlimannii* has been isolated and for which transstadial and transovarial transmission have been assessed. This *Rickettsia* was first isolated from *H. marginatum* ticks from Morocco in 1997, and its pathogenicity to humans was recently assessed in a patient with a boutonneuse fever–like syndrome. Some authors claimed there was evidence that this *Rickettsia* species could be replacing *R. conorii* as the etiologic agent of boutonneuse fever in northwestern Spain. Similarly, no positives to *R. conorii* were found in this study, even with the reasonably high number of *R. sanguineus* analyzed (146), all feeding on animals, mostly dogs. Whether these results imply that the etiology of boutonneuse fever is changing needs to be confirmed in patients, although, at least is areas of central and northwestern Spain, *R. conorii* seems not to play an active role. Alternatively, as the diagnosis of boutonneuse fever in the past was mostly clinical and serologic, we have no relevant microbiological data about the etiologic agent involved in the human cases historically described in the area and, consequently, they could have been related to *Rickettsia* species other than *R. conorii*. Even though *H. marginatum* is not prevalent in central Spain (2.9% of the total of feeding ticks collected), 8 of 13 specimens analyzed were shown to carry *R. aeschlimannii* DNA, which implies a high risk for human disease in the case of a *H. marginatum* bite.

The human pathogen *R. sibirica* (strain mongolotimonae) was detected in six specimens of two different tick species (*R. pusillus* and *R. bursa*). Earlier studies mainly associated this *Rickettsia* with *Hyalomma* ticks, although it has also been described from *R. pusillus* in Portugal. In our study, it was found in four specimens of *R. pusillus* and two of *R. bursa*, all feeding on animals. To our knowledge, this is the first report of *R. sibirica* strain mongolotimonae in *R. bursa*. Because the *R. bursa* specimens positive to this *Rickettsia* species were all collected from animals, their vectorial role could not be directly inferred, and it needs to be further assessed. These findings confirm that this *Rickettsia* strain is circulating in Spain, where human cases have recently been described.
of the same species), as well as in Africa and the United States, among other countries. In our study, R. massiliae was found in different species of the genus Rhipicephalus (R. sanguineus, R. pusillus, and R. bursa), which increases the range of reported species carrying this pathogen.

The detection of R. felis in R. sanguineus, although unexpected, is not surprising. It is well known that the vector of R. felis, the cat flea Ctenocephalides felis, often bites other animals such as dogs, which are the main host for R. sanguineus. In addition, previous studies have reported the presence of R. felis in hard ticks such as Amblyomma cajennense and R. sanguineus, and these findings are not necessarily related to a vectorial role. Finally, R. helvatica was only found in one I. ricinus specimen, in accordance with the specific association of this bacterium with that tick species.

In summary, seven Rickettsia have been detected in six ixodid tick species in central Spain, with an overall infection rate of 19%. Given the proven transstadial and transovarial transmission of the majority of the Rickettsia species found in this study in several tick species, endemic focus for the transmission of Rickettsiae in central Spain has been shown and, therefore, the risk of infection by Rickettsia after a tick bite is high.

Borrelia burgdorferi s.l., the agent of Lyme disease, is widespread in the Northern Hemisphere, and it has been detected in a wide range of hard ticks, although species of the I. ricinus complex are considered the main vectors. The northern fringe of Spain, where I. ricinus is the most abundant tick species, has been shown to be endemic for B. burgdorferi s.l. In contrast, in central Spain, where the density and diversity of tick species is different, I. ricinus was found occasionally and only in one of the six areas tested. The eight specimens captured represent 0.8% of the QT collected in this study; of them, two were positive to Borrelia. Other tick species were found to be positive for this agent and, in particular, H. lusitanicum accounted for the largest number of positives (23 specimens, 3.3% of questing H. lusitanicum). However, from the 23 H. lusitanicum—positive specimens, we could not fully characterize 7 of them. Consequently, with the available data, we were not able to confirm whether these seven samples were B. burgdorferi or a closely related organism. A. afflicti was not found, suggesting that competent reservoirs for this genospecies are not present in the study area. A similar situation was found in northern Spain, where rodents are not playing a role as reservoir hosts for B. burgdorferi s.l. However, the presence of B. burgdorferi s.s. and B. garinii, associated in other studies with birds, suggest that rodents could not play a role in maintaining these genospecies in nature in the study area. The fact that immature stages of H. lusitanicum also often feed on birds supports this hypothesis. No ticks infected by Borrelia were found feeding on animals, which could be related to a clearance effect of feeding on incompetent reservoirs. Consequently, the potential risk of acquiring the disease by tick bite in central Spain is low, except in one area in northern Madrid, where I. ricinus was found in this study with a high percentage of B. burgdorferi infection, implying a moderate risk for humans.

Ixodes ricinus is the proven vector of A. phagocytophilum in Europe. However, there have been descriptions throughout Spain of different tick species found positive to this agent by PCR, including H. lusitanicum, D. marginatus, and R. bursa. In our study, the same three tick species have been found apparently infected by A. phagocytophilum, in addition to one specimen of R. pusillus, species that seems to be only scarcely infected. The combined msp2-based PCR/RLB should provide a high specificity, and in our hands, it did not amplify A. marginale and A. bovis (data not shown). However, this was not assessed with a wider panel of Anaplasma species. Given all these, whether the organisms detected are in fact A. phagocytophilum or an uncharacterized, closely related species is still to be determined. In any case, without experiments on vectorial competence for these tick species, and on the determination of the specific genotypes of A. phagocytophilum detected, the epidemiologic significance of these findings must be measured carefully.

Francisella tularensis subsp. holarctica causes outbreaks of tularemia in endemic areas throughout Europe. In Spain, two outbreaks have occurred in Castilla-León in the last 10 years. The reservoirs of the bacterium are not well established, and mosquitoes and ticks are suspected to contribute in maintaining the agent between outbreaks in aquatic and terrestrial cycles, respectively. Although tick bite is not the main route for the transmission of tularemia in Europe, three cases of transmission associated with Dermacentor ticks have been described in Spain. The results of this study showed low infection rates in D. marginatus, R. sanguineus, and H. lusitanicum (1.4, 0.6, and 0.1, respectively), and sporadic cases of human tularemia have not yet been described in the study area. In any case, because transovarial transmission of F. tularensis has been shown in D. marginatus, this species could play an important role in the maintenance of the pathogen in central Spain, where surveillance methods should be implemented for a rapid identification of endemic areas and human cases.

Although the role of ticks in the transmission of Bartonella has not been confirmed, Eskow and others have reported a human co-infection by B. henselae and B. burgdorferi attributed to tick exposure, and Bartonella species have been detected in ticks from North America, Europe, and Asia. However, as in other studies, we have found a lack of Bartonella in the ticks analyzed, indicating that, in our study area, ticks do not seem to play a role in the wild cycle of this bacteria.

Finally, among the mixed infections found (0.5% of ticks analyzed), the majority corresponded to co-infections by A. phagocytophilum and B. burgdorferi. This has been often described in I. ricinus, and in this study was found in six specimens of QT H. lusitanicum (0.8%). I. ricinus was found only scarcely in our sampling sites, whose climatologic conditions favored the predominance of H. lusitanicum, a tick species of dry climates. It would be possible that, in the absence of I. ricinus, H. lusitanicum could be taking its place in maintaining both bacteria in nature. The immature stages of H. lusitanicum mostly feed on rodents and birds, both known to carry A. phagocytophilum and B. burgdorferi. Given our results in questing (i.e., unfed) adult H. lusitanicum, we can hypothesize about its involvement in maintaining both pathogens in nature.

In summary, in terms of infection and tick abundance, H. lusitanicum seems to be the most significant tick species in the area, carrying three of the five agents tested. Fortunately, in terms of public health, this tick is not anthropophilic. On the contrary, D. marginatus, frequently found here infected with Rickettsia spp., and, to a lesser extent, F. tularensis, often bite humans and could play a principal role in tick-borne bacteria transmission. In this study, the high number of QT and TA ticks tested throughout the year has provided us with data
about tick-borne and related bacterial pathogens that represent a risk for humans and animals in central Spain, highlighting a high diversity of both ticks and pathogens distribution among areas in Spain.

Received October 14, 2008. Accepted for publication March 13, 2009.

Acknowledgments: We thank Isabel Rodríguez-Moreno, Manuela Rodríguez-Vargas, and Cristina García-Amil for excellent technical support and Frank M. Hodgkins for reviewing the English version of this manuscript.

Financial support: A.T. was supported by a fellowship from Fondo de Investigación Sanitaria (FIS) P051374. This work was also supported by FIS P0509019, INIA FAU2006-00002-C04-04, and EU Grant GOCE-2003-010284 EDEN and is catalogued by EDEN Steering Committee as EDEN0126 (www.eden-fp6project.net).

Disclaimer: The content of this publication is the sole responsibility of the authors and does not necessarily reflect the views of the European Commission.

Authors’ addresses: Álvaro Toledo, Raquel Escudero, Isabel Jado, Horacio Gil, and Pedro Anda, Laboratorio de Espiroquetas y Patógenos de las Infecciones de la Investigación Sanitaria (FIS) PI051374. This work was also supported by FIS P0509019, INIA FAU2006-00002-C04-04, and EU Grant GOCE-2003-010284 EDEN and is catalogued by EDEN Steering Committee as EDEN0126 (www.eden-fp6project.net).

REFERENCES


